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Infraspecific chemical variability of the leaf essential oil of *Juniperus phoenicea* var. *turbinata* from Portugal

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Abstract

The essential oil composition of 68 individual plants of *Juniperus phoenicea* from Portugal was investigated by GC, GC-MS and ^{13}C NMR. α-Pinene, β-phellandrene, α-terpinyl acetate and myrcene were found to be the main constituents. Botanical and chemical data as well as phytogeographical distribution indicate *J. phoenicea* var. *turbinata* as the unique subspecies occurring in Portugal. Nevertheless, this taxon exhibits chemical polymorphism. The results of the oil compositions were processed by hierarchical clustering and principal component analysis allowing to establish three groups of essential oils differentiated by the content of α-pinene, β-phellandrene and α-terpinyl acetate.

Keywords: *Juniperus phoenicea* var. *turbinata*; Cupressaceae; Essential oil; GC; GC–MS; ^{13}C NMR; Infraspecific variability

1. Introduction

Juniperus phoenicea L. is a native species of Portugal but some taxonomic problems subsist at subspecific level. Franco (1986) reported the occurrence in

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Portugal of a unique subspecies, *J. phoenicea* subsp. *phoenicea* L., while Costa et al. (1993) mention only *Juniperus phoenicea* subsp. *turbinata* (Guss.) Parl. Nyman. (= *J. phoenicea* subsp. *lycia* Auct. = *J. turbinata* Guss.). A chemotaxonomic study of *Juniperus phoenicea* based on the ratio of the leaf prodelphinidine/procyanidine led a new taxon, *J. phoenicea* subsp. *eu-mediterranea* Lebr. & Tiv. (Lebreton and Thivend, 1981), considered later as the unique subspecies occurring in Portugal (Lebreton, 1983). The controversy about the taxonomy of the portuguese populations of *Juniperus phoenicea* was partially solved by the comparison of the essential oil composition from var. *turbinata* and subsp. *eu-mediterranea* (Adams et al., 1996). The similar compositions of the oils of var. *turbinata* from Spain and subsp. *eu-mediterranea* from Portugal, with important amounts of α-pinene, β-phellandrene and α-terpinyl acetate, lead the authors to conclude that var. *turbinata* and subsp. *eu-mediterranea* are conspecific but different from *J. phoenicea* var. *phoenicea* whose oil has higher quantities of α-pinene and lower of β-phellandrene.

Studies on the composition of the essential oil of *J. phoenicea* from Portugal (Cavaleiro et al., 1996) showed that among portuguese populations other oil composition patterns were present. Distinct compositions were also found in the Corsican populations of *J. phoenicea* (Rezzi et al., 2001).

The aim of this study was to evaluate, analyzing isolated plants from a large number of portuguese populations, the significance of the variability of the leaf essential oil and discuss its contribution for the taxonomy and characterization of infraspecific variability of *J. phoenicea*.

2. Materials and methods

2.1. Plant material

Sixty-eight samples of single plants were collected in the areas of distribution of *J. phoenicea,* specially in the littoral south and center of Portugal: Costa Vicentina-Vila do Bispo (samples 1–14), Sudoeste Alentejano-Porto Covo (15–24), Noroeste Alentejano-Melides (25–28), Península de Troia-Troia (29–43), Península de Setúbal-Serra da Arrábida (44–58), Cabo Mondego-Gala (59–62, 68) and Guadiana interior-Mértola (63–67).

Voucher specimens were deposited in the Herbarium of the Instituto Botânico of the University of Coimbra (COI).

2.2. Sampling and essential oils isolation

Leaves were collected during the period of May–September of 1995–97, when the plants exhibit a majority of mature berries, and submitted to water distillation for 3 h using a Clevenger-type apparatus. Essential oil yield, ranged between 0.2% and 0.9% (v/w, from fresh material).

2.3. GC, GC/MS and ^{13}carbon NMR analyses

Analytical GC was carried out on an Hewlett Packard 6890 gas chromatograph with HP GC ChemStation Rev. A.05.04 data handling system, equipped with a single injector and two flame ionization detectors (FID). Two fused silica capillary columns with different polarities were used: SPB-1 (polydimethylsiloxane 30 m × 0.20 mm i.d., film thickness 0.20 μm), and SupelcoWax 10 (polyethyleneglycol 30 m × 0.20 mm i.d., film thickness 0.20 μm). Oven temperature program: 70°C–220°C (3°C/min), 220°C (15 min); injector temperature: 250°C; carrier gas: helium, adjusted to a linear velocity of 30 m/s; splitting ratio 1 : 40; detectors temperature: 250°C.

Relative amounts of individual components were calculated based on peak areas without FID response factor correction. Retention indices were determined by linear interpolation relative to retention times of a series of *n*-alkanes.

GC-MS was performed with an Hewlett Packard 6890 gas chromatograph, with a HP1 fused silica column (polydimethylsiloxane 30 m × 0.25 mm i.d., film thickness 0.25 μm), interfaced with an Hewlett Packard Mass Selective Detector 5973 operated by HP Enhanced ChemStation software, version A.03.00. GC parameters as above; interface temperature: 250°C; MS source temperature: 230°C; MS quadrupole temperature: 150°C; ionization energy: 70 eV; ionization current: 60 μA; scan range: 35–350u; scans/s: 4.51.

^{13}C-NMR spectra were recorded on a Bruker AC 200 Fourier Transform Spectrometer, operating at 50.323 MHz, equipped with a 10 mm probe in deuterated chloroform (around 200 mg of oil in 2 ml of $CDCl_3$) with all shifts referred to internal tetramethylsilane (TMS). Parameters: pulse width (PW): 5.0 μs (flip angle 45°); acquisition time: 1.3 s and relaxation delay D_1: 2 s (total recycling time 3.3 s) for 32 K data table with a spectral width (SW) of 12,500 Hz (250 ppm); Composite Phase Decoupling (CPD) of the proton band; digital resolution: 0.763 Hz/pt; 5000 scans were accumulated for each sample. An exponential multiplication of the free induction decay with the line broadening of 1.0 Hz was applied before Fourier transformation.

The constituents of the essential oils were identified on the basis of their GC retention indices (RI), and by matching their 70 eV mass spectra with our own data and reference libraries (Adams, 1995; Joulain and König, 1998; McLafferty and Stauffer, 1989). Major compounds were also identified by ^{13}C-NMR. The identification was performed by computer-aided analysis of the ^{13}C-NMR spectrum of the total oil, by comparing the signals obtained with those of pure compounds included in a library created in our laboratory (Tomi et al., 1995; Rezzi et al., 2001). This ^{13}C-NMR technique has proved to be useful for the identification of ambiguous components (detection limit: 0.5%) which are poorly separated by GC or insufficiently elucidated by mass spectra and retention indices.

2.4. Data analysis

The compositional data of 68 investigated samples of *J. phoenicea* var *turbinata* from Portugal and data of four reference samples obtained from literature (Adams

et al., 1996)—two samples of *J. phoenicea* var. *phoenicea* (cases 69–70), one sample of *J. phoenicea* var. *turbinata* (case 71) and one sample of *J. phoenicea* ssp. *eu-mediterranea* (case 72)—were submitted to Multivariate Statistic Analysis accomplished by SPSS (Superior Performing Software Systems, Inc.) 10.0. Only the 40 constituents over 0.5% were used as variables for analysis.

Data was subject to a hierarchical clustering using average linkage with square Euclidean distance measure and factor analysis using the Principal Components extraction method (PCA). The aptitude of the complete correlation matrix was checked by the Kaiser Meyer-Olkin criterion (KMO = 0.519). After PCA, another hierarchical clustering concerning to the two extracted principal components was performed.

3. Results and discussion

Sixty-eight components amounting from 86.9 to 99.8% of the total oil were identified (Table 1). α-Pinene, β-phellandrene, α-terpinyl acetate, and myrcene were found to be the main constituents. From hierarchical clustering dendrogram (Fig. 1), the 68 samples of *J. phoenicea* var. *turbinata* from Portugal as well as the reference samples of *J. phoenicea* var. *turbinata* and *J. phoenicea* ssp. *eu-mediterranea* were classified in three significant clusters (clusters A, B, and C). Reference cases of *J. phoenicea* var. *phoenicea* were classified apart in another cluster (cluster D).

PCA reduced the 40 variables to two principal components representing 95.2% of the total variance, having α-pinene, β-phellandrene and α-terpinyl acetate the highest coefficient factors. The hierarchical classification relative to the two extracted components confirmed clustering based in the original variables.

PCA, together with both hierarchical classifications (Fig. 2) allowed establish four types of essential oils characterized in the base of their α-pinene, β-phellandrene and α-terpinyl acetate contents.

Two clusters (A and B) were differentiated in the basis of their α-pinene, β-phellandrene and α-terpinyl acetate ratios: the oils of cluster A (63.2% of the samples) are characterized by α-pinene (average = 44.3%, SD = 6.4), β-phellandrene (average = 22.7%, SD = 3.5) and α-terpinyl acetate (average = 6.9%, SD = 2.3), the ratio α-pinene:β-phellandrene is close to 2 : 1; essential oils of cluster B (32.4% of the samples) are also characterized by α-pinene (average = 27.8%, SD = 4.8), β-phellandrene (average = 28.8%, SD = 3.2) and α-terpinyl acetate (average = 10.5%, SD = 3.1) but the ratio α-pinene:β-phellandrene is close to 1. The composition of the oils from cluster B is similar to those reported for oils of *J. phoenicea* var. *turbinata* from Spain (reference case 71) (Adams et al., 1996), while composition oils from cluster A is similar to those *J. phoenicea* subsp. *eu-mediterranea* (reference case 72) (Adams et al., 1996) and those of plants from *J. phoenicea* var. *turbinata* growing in Corsica (Rezzi et al., 2001). The essential oils of cluster C (4.4% of the samples) are dominated by α-pinene (average = 81.5%, SD = 4.8). This extremely high content of α-pinene was not yet reported for *J. phoenicea* leaf oil but only described for oils

Table 1
Composition of the essential oils of the three clusters of *J. phoenicea* var. *turbinata* from Portugal[a]

Components	RI[b]	% Average (range)		
		Cluster A	Cluster B	Cluster C
Tricyclene	921	0.1 (0–0.3)	0.1 (0–0.2)	0.2 (0.2–0.3)
α-Pinene[c]	930	44.3 (35.6–63.2)	27.8 (18.1–35.7)	81.5 (76.3–85.8)
α-Fenchene	941	0.2 (0–0.9)	0.1 (0–0.9)	0.1 (0–0.4)
Camphene	941	0.3 (*t*–0.8)	0.3 (0.1–0.7)	0.6 (0.4–1.0)
Sabinene	964	0.1 (*t*–0.4)	0.3 (*t*–0.4)	0.1 (0.1–0.2)
β-Pinene[c]	970	1.3 (0.9–1.8)	1.2 (0.6–1.8)	1.2 (0.8–1.4)
Myrcene[c]	980	6.3 (4.1–7.9)	7.1 (5.3–11.0)	2.5 (2.2–2.8)
α-Phellandrene[c]	997	3.6 (1.1–4.9)	4.1 (2.1–5.4)	0.1 (0.1–0.2)
Δ-2-Carene[c]	998	0.6 (*t*–1.8)	0.9 (*t*–2.4)	*t* (0–*t*)
Δ-3-Carene	1005	0.2 (0–7.8)	0.3 (0–6.2)	*t* (0–0.1)
α-Terpinene	1010	0.4 (*t*–1.4)	0.4 (0.1–0.7)	*t* (0–0.1)
p-Cymene[c]	1011	1.0 (0.6–2.2)	1.2 (0.8–2.3)	0.2 (0.2–0.3)
Limonene[c]	1020	2.3 (0.3–5.7)	2.5 (1.4–4.0)	1.4 (0.9–2.3)
β-Phellandrene[c]	1020	22.7 (13.4–31.5)	28.8 (24.9–38.0)	0.4 (0.4–0.5)
E-β-Ocimene	1035	*t* (0–0.1)	—	*t* (0–*t*)
γ-Terpinene[c]	1047	0.3 (0.1–0.4)	0.3 (*t*–0.6)	0.3 (0.3–0.4)
Fenchone	1066	*t* (0–0.19)	*t* (0–0.1)	0.1 (0–0.2)
Cymenene	1071	0.1 (0–0.2)	0.1 (0–0.2)	*t* (0–0.1)
Terpinolene[c]	1077	1.3 (0.6–1.8)	1.6 (1.2–1.9)	0.5 (0.5–0.5)
Linalool	1082	0.1 (0–0.4)	0.2 (0–0.7)	0.5 (0.2–0.9)
α-Fenchol	1098	*t* (0–0.1)	*t* (0–0.1)	0.1 (0–0.1)
α-Campholenal	1104	0.1 (0–0.3)	*t* (0–0.1)	0.1 (0–0.1)
Z-*p*-Menth-2-en-1-ol	1106	0.3 (0–0.7)	0.5 (0–1.2)	0.1 (0–0.4)
Camphor	1119	*t* (0–0.1)	*t* (0–0.1)	—
E-*p*-Menth-2-en-1-ol	1120	0.4 (0–0.9)	0.4 (0–0.9)	—
cis-Verbenol	1126	0.1 (0–0.3)	0.1 (0–0.3)	0.1 (0–0.2)
Isoborneol	1138	0.1 (0–0.4)	*t* (0–0.2)	0.1 (0–0.2)
p-Mentha-1,5-dien-8-ol	1143	0.1 (0–0.3)	0.1 (0–0.3)	—
Cryptone	1152	0.1 (0–0.3)	0.1 (0–0.4)	—
p-Cymene-8-ol	1158	0.1 (0–0.4)	0.1 (0–0.3)	*t* (0–0.1)
Terpineol-4	1158	0.1 (0–0.3)	0.2 (*t*–0.3)	0.2 (*t*–0.4)
α-Terpineol	1169	0.7 (0–1.6)	0.9 (0.5–1.4)	0.5 (0.3–0.8)
cis-Piperitol	1177	0.1 (0–0.3)	0.2 (0–0.4)	0.1 (0–0.3)
trans-Piperitol	1187	0.1 (0–0.4)	0.2 (0–0.4)	*t* (0–0.1)
cis-Carveol	1195	*t* (0–0.2)	*t* (0–0.1)	–
Fenchyl acetate	1204	0.1 (0–0.2)	*t* (0–0.2)	0.2 (0.2–0.3)
Citronellol[c]	1210	0.3 (0–1.5)	0.6 (0–2.2)	*t* (0–0.1)
trans-Carveol	1212	*t* (0–0.2)	*t* (0–0.2)	–
Piperitone	1222	0.2 (0–0.6)	0.3 (0–0.6)	–
Geraniol	1233	*t* (0–0.2)	*t* (0–0.4)	0.1 (0–0.2)
3-Decene-1-ol	1238	0.2 (0–0.9)	0.4 (0–0.9)	0.2 (0–0.5)
Linalyl acetate	1240	0.1 (0–0.2)	0.1(0–0.2)	0.4 (0–1.1)
Isopulegyl acetate[c]	1254	0.3 (0–1.4)	0.2 (0–1.6)	—
Bornyl acetate	1267	0.3 (0–0.6)	0.4 (0–1.2)	0.7 (0.3–0.9)
α-Terpenyl acetate[c]	1331	6.9 (2.2–11.9)	10.5 (3.1–15.0)	0.3 (0.1–0.4)
β-Elemene	1382	*t* (0–0.3)	0.1 (0–0.5)	*t* (0–0.1)
E-Caryophyllene	1408	0.1 (0–0.3)	0.2 (0.1–0.6)	0.2 (0.1–0.3)

Table 1 (*continued*)

Components	RI[b]	% Average (range)		
		Cluster A	Cluster B	Cluster C
γ-Elemene	1422	*t* (0–0.1)	*t* (0–0.2)	—
α-Humulene	1442	*t* (0–0.2)	*t* (0–0.2)	0.1 (0–0.2)
Germacrene D	1467	0.2 (0–0.6)	0.3 (0–1.1)	0.3 (0.1–0.4)
α-Muurolene	1486	0.1 (0–0.4)	0.1 (0–0.4)	0.1 (0–0.2)
γ-Cadinene	1498	0.1 (0–0.3)	0.1 (0–0.4)	0.2 (*t*–0.2)
cis-Calamelene	1502	*t* (0–0.1)	*t* (0–0.9)	—
δ-Cadinene[c]	1508	0.3 (0.1–0.8)	0.5 (0.2–0.9)	0.6 (0.2–0.8)
Elemol	1526	0.3 (0–0.7)	0.4 (0.2–0.8)	0.4 (0.2–0.5)
Germacrene B	1540	0.2 (0–0.9)	0.3 (0-0.9)	*t* (0-0.1)
Nerolidol	1545	*t* (0-0.2)	*t* (0–0.4)	0.2 (0–0.5)
Caryophyllene oxide	1557	0.1 (0–0.3)	0.1 (0–0.3)	*t* (0–0.1)
α-Cedrol	1575	—	0.4 (0–5.4)	—
Humulene epoxide	1582	*t* (0–0.1)	0.0 (0–0.1)	—
γ-Eudesmol	1607	*t* (0–0.2)	0.1 (0–0.3)	—
α-Cadinol	1616	0.1 (0–0.3)	0.2 (0–0.5)	0.2 (0–0.5)
β-Eudesmol	1620	*t* (0–0.3)	0.1 (0–1.3)	0.2 (0–0.3)
τ-Muurolol	1627	0.3 (0–0.7)	0.5 (0.2–1.5)	0.6 (0.1–1.3)
Sandaracopimaradiene	1941	*t* (0–0.3)	*t* (0–0.3)	*t* (0–*t*)
Manoyl oxide[c]	1964	0.1 (0–1.3)	0.2 (0–1.0)	*t* (0–0.1)
Abietatriene	2018	*t* (0–0.2)	*t* (0–0.4)	0.1 (0–0.2)
Totarol[c]	2253	*t* (0–0.6)	0.2 (0–1.0)	0.3 (0–0.6)
Monoterpene hydrocarbons		89.6 (84.1–94.6)	76.8 (60.1–88.1)	85.0 (74.7–93.7)
Oxygen containing monoterpenes		3.3 (1.8–4.6)	15.3 (7.9–23.8)	10.5 (3.6–17.3)
Sesquiterpene hydrocarbons		1.5 (0.6–2.0)	1.6 (0.4–4.9)	1.1 (0.3–2.2)
Oxygen containing sesquiterpenes		1.7 (0.4–2.6)	2.0 (0.4–8.5)	0.9 (0–1.8)
Others		0.5 (0.1–0.8)	0.9 (0–2.3)	0.4 (0–1.8)
Total identified		96.5 (93.8–99.7)	96.5 (88.0–99.6)	97.8 (86.9–99.8)

[a] = traces (<0.05%).
[b] Retention index on the SPB-1 column.
[c] Identity confirmed by ^{13}C-NMR.

isolated from berries (Proenca da Cunha et al., 1977; De Pascual Teresa et al., 1981; Vidrich and Michelozzi, 1993; Falchi Delitala, 1980; Lawrence, 1989).

Composition patterns corresponding to clusters A and B are present in all the collection areas with a similar distribution, excepting Península de Troia and Península de Setúbal, where oils of cluster A are dominant. Composition pattern corresponding to cluster C (three samples) was only detected in Costa Vicentina, Sudoeste Alentejano and Cabo Mondego.

No conspicuous botanical differences were observed among the plants and, in other hand, plants with distinct oil composition are present in each population and from different habitats. The littoral distribution, the morphological features and the chemical data, including the comparison with the reference samples of *J. phoenicea* var *turbinata*, *J. phoenicea* subsp *eu-mediterranea* and *J. phoenicea* var. *phoenicea*, lead us to consider *Juniperus phoenicea* var. *turbinata* the unique variety spontaneous

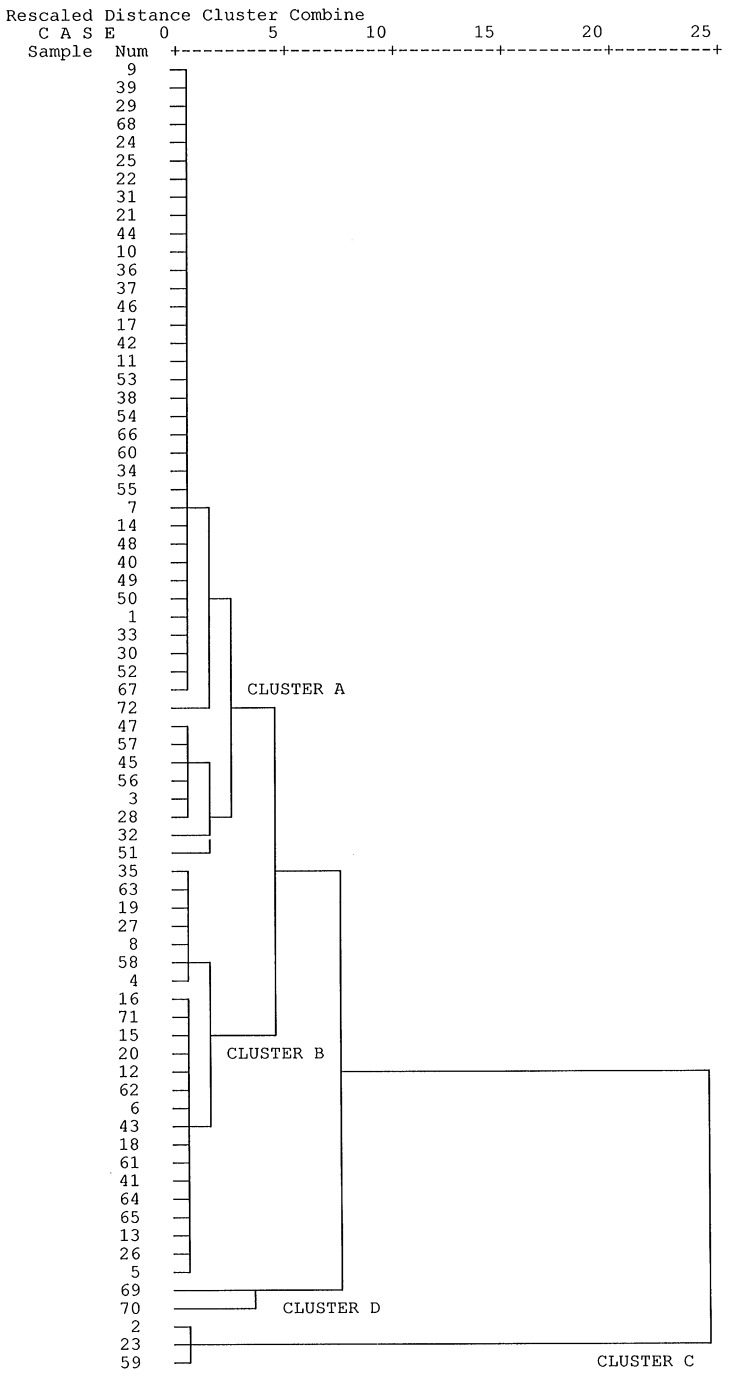

Fig. 1. Dendrogram from the hierarchical cluster analysis based in the original variables.

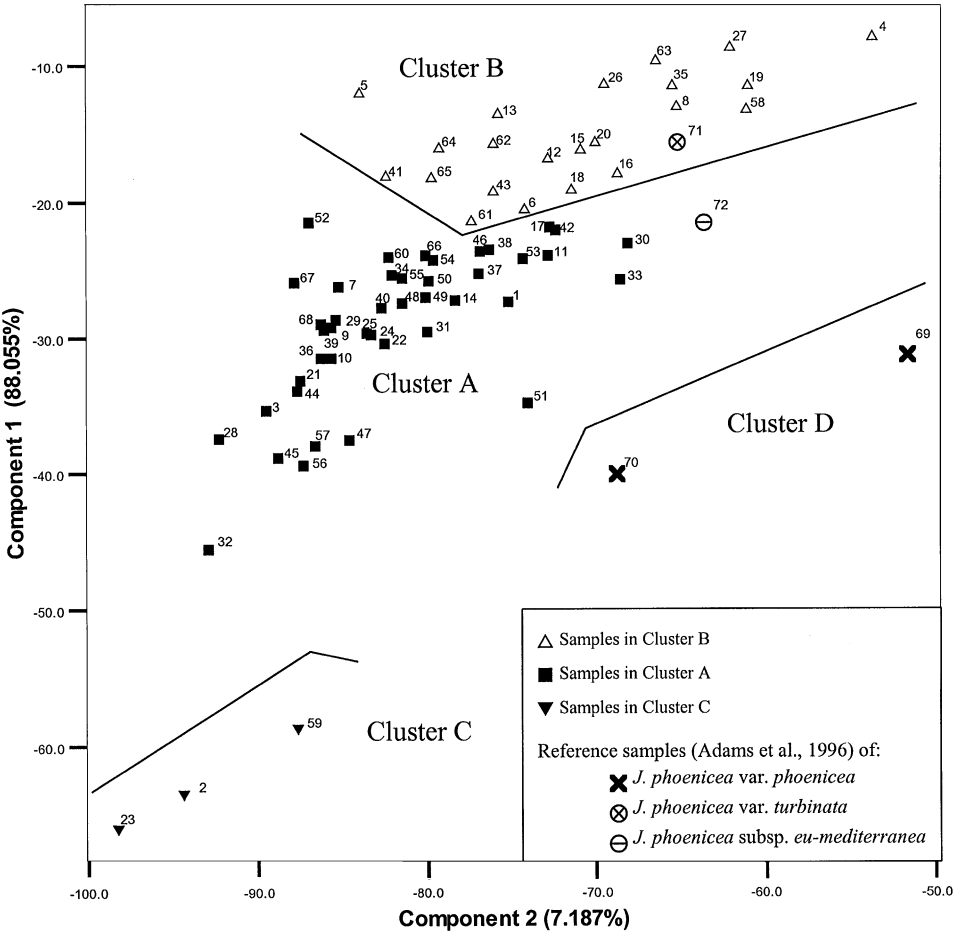

Fig. 2. Scatterplot of the samples for the two principal components extracted in PCA.

from Portugal. Nevertheless it appears from our results that the portuguese *J. phoenicea* var. *turbinata* exhibit an infraspecific chemical variability with two dominant types of essential oils.

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